

Bivalve *Mytilus* spp. Toxicity Test

1.0 OBJECTIVE

The purpose of the *Mytilus* spp. 48-hr static embryo-larval development toxicity test is to determine the concentration of a test substance that reduces development of mussel embryos. Abnormal shell development and mortality indicate toxicity. Test results indicate whether a sample has produced statistically significant abnormal development relative to a control or reference sample.

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate SOP (MPSL SOP 1.3).

2.1 Test Initiation

- Volumetric flasks and pipettes for reference toxicant dilutions
- Squeeze bottles
- Pipettes, automatic and manual
- Sedgewick-Rafter counting cell
- Mixing Plunger
- Graduated cylinders
- Constant temperature room at 15°C
- Gloves and appropriate safety gear (see MPSL lab safety manual)

2.2 Test Termination

- Inverted microscope
- Data sheets
- Formaldehyde
- Fume hood
- Gloves and appropriate safety gear (see MPSL lab safety manual)

2.3 Water Quality

- Meters, probes and spectrophotometer for measuring pH, dissolved oxygen, salinity, and ammonia
- Thermometers (glass spirit thermometer and logger)
- Graduated pipettes and 30-ml glass vials
- Gloves and appropriate safety gear (see MPSL lab safety manual)

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2.4 Dilution Water

For tests at Marine Pollution Studies Laboratory, dilution water is ambient Granite Canyon seawater, filtered to 1 μm , at ambient salinity (33-34‰). This water may be diluted to 28‰ with distilled water or Nanopure water for use in preparing embryos for toxicity tests, and for diluting test solutions.

To prepare 28‰ water, mix about 8.4 liters of seawater (at 34‰) with 1.6 liters of distilled water to get 10 liters of 28‰ water. Use this recipe only as a guide, since seawater salinity may vary. Measure the actual salinity, adding and mixing seawater or distilled water as needed until salinity reaches $28 \pm 1\text{‰}$.

3.0 EXPERIMENTAL DESIGN

This laboratory toxicity test consists of five replicate scintillation vials for each sample. Samples are occasionally diluted into several concentrations, also replicated five times. Vials are arranged randomly, and each receives approximately 200 mussel embryos. Embryos are allowed to develop for 48 hours to the prodissoconch I larval stage. The quality of test embryos and testing conditions is determined through concurrent testing of reference toxicants (positive controls), seawater control, and brine control (negative controls). Testing of reference sites is recommended to demonstrate the suitability of test porewater and elutriate in the absence of toxic contaminant concentrations. Dissolved oxygen, pH, salinity, ammonia, and temperature are measured at the beginning and end of the exposure.

4.0 PREPARATION OF SAMPLES FOR TESTING

Label scintillation vials as indicated on the randomization sheet generated for the test. Label another set of vials, three each with the sample number of each sample, to be used for water quality measurements at the end of the test. Determine the salinity of the test solutions. Be sure to stir all samples before measuring salinity, and measure salinity immediately after stirring. Sample salinities more than 2‰ below test salinity are adjusted using hypersaline brine made from frozen seawater or artificial salts. Check the pH of brine. If necessary, adjust the brine pH by adding acid or sodium hydroxide until it is between 7.5-8.5.

Using the random number sheet, aliquot 10 mL of sample to scintillation vials and water quality containers. Minimize sample exposure to sunlight (never leave samples in direct sunlight), and schedule loading times to avoid prolonged sample exposure to temperatures above 15°C.

5.0 CONTROLS

5.1 Seawater and Brine Controls (Negative Controls)

A seawater control consisting of 1- μm filtered Granite Canyon water (optionally adjusted to 28‰) should accompany each batch of samples. If any salinity adjustments were made a brine controls must also be prepared.

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The brine control must contain the same amount of brine as the lowest salinity sample (Refer to Salinity Adjustment SOP).

5.2 Reference Toxicant Tests (Positive Controls)

Conduct a concurrent reference toxicant test each time a mussel development test is conducted (standard procedure). The reference toxicant test uses identical test conditions as those used in testing samples, and provides data on the relative sensitivity of each batch of mussel embryos.

Conduct a concurrent reference toxicant test on a monthly basis. The reference toxicant test is for a similar exposure time, and provides data on the relative sensitivity of each batch of mussel gametes.

Prepare a stock solution of 100,000 µg Cd/L by weighing 0.1630 g of cadmium chloride (CdCl₂), and pouring the weighed solid into one liter of distilled water in a plastic volumetric flask. Reference toxicant solutions should be four replicates of 0, 1, 1.8, 3.2, 5.6, and 10 µg/L. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare concentrations according to dilution schedule. Start with the control solutions and progress to the highest concentration to minimize contamination. Use plastic volumetric flasks and beakers to reduce loss of cadmium to container walls.

6.0 TEST INITIATION

Five replicates are used for all treatments of samples. Each replicate contains 10 mL of test solution. Initial density containers should also be prepared with 10 mL seawater. These containers will be preserved immediately after inoculation to determine the initial density of cleaved eggs added to the test.

6.1 Collection and Preparation of Gametes

Viable gametes must first be collected, and then diluted to the appropriate concentration for fertilization. Fertilized embryos must then be counted so the correct number of embryos can be delivered to each test container. Follow the worksheet for guidance.

Mussels are cleaned by scraping shells with a wire brush to break off barnacles. Place mussels in empty culture trays. Fill trays with ambient salinity water that is ambient + 10°C. Wait until mussels begin to release gametes. As mussels begin to spawn, remove them from the spawning tray and place them in crystallizing dishes containing water at test salinity and temperature. Allow them to continue spawning. The period between cycles should be approximately one hour.

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6.2 Selection and Pooling of Gametes

Eggs must be inspected to determine shape and uniformity. Eggs should have a round to ovoid shape. Do not use eggs from females that release eggs in clumps. If the viability of a female's eggs is questionable, and no other eggs are available, place one drop of eggs onto well slide and add a small amount of sperm to test fertilization. Check for fertilization micropile after 15-25 minutes. If micropile does not form, do not use eggs. Sperm should be checked to verify motility.

6.3 Fertilization of Gametes

Eggs from two or more female mussels are combined and poured through a 90 µm screen. Dilute the egg suspension until the color of the solution is a pale orange (~20-50 eggs/mL). After the sperm motility has been verified by microscopic examination, the sperm suspension should be passed through a 25-µm screen into a glass beaker. Sperm density must be 10^5 to 10^7 sperm per mL in the final mixture. Precise sperm counts are unnecessary after one gains experience visually estimating sperm density.

Eggs and sperm should be combined in a beaker and held at 15°C. After approximately 5 minutes, take a sample of the egg suspension and verify sperm motility and quantity. When each egg has approximately 15 sperm around its outer (visible) edge, rinse the egg suspension through a 25-µm screen to remove the sperm. Pour the rinsed eggs into a beaker of water.

After approximately one hour, and approximately every 15 minutes thereafter, take a sample of embryos to monitor cleavage. The test can be initiated when there are greater than 95% cleaved cells. While monitoring cleavage, adjust the density of the egg suspension to approximately 200 embryos per 100 µL. The test should be inoculated within 4 hours after fertilization.

6.4 Inoculating the Test

Test containers should be inoculated with 100 µl of the embryo mixture. This will yield an embryo density of approximately 20 embryos/ml. After test inoculation, initial density containers should be preserved and counted immediately.

7.0 TEST TERMINATION

To terminate the test, use a toxic dispenser or disposable pipette, add 0.25 mL 37% buffered formalin to each sample to give a final formalin concentration of 2.5%. Gently shake containers to mix. Test containers may now be capped and stored for later evaluation.

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8.0 DATA COLLECTION AND TABULATION

8.1 Counting Test Chambers

After the 48-hour incubation, and subsequent preservation, count all of the embryos present in the sample. Use a hand counter to count the total number of normal larvae. D-shaped shells with an obvious flat hinge, no noticeable dents around the border, and tissue present define normal larvae. Initial density samples should be counted immediately after inoculating the test. Count all of the cleaved eggs in the container.

9.0 DATA HANDLING AND TEST ACCEPTABILITY

This toxicity test procedure is considered unacceptable if the percentage of normal alive larvae is below 70% of test controls. Brine controls and reference toxicant controls must also be greater than or equal to 70%. Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures range from 14°C to 16°C; acceptable salinities range from 26‰ to 30‰; acceptable dissolved oxygen concentrations range from 5.09 to 8.49 mg/l.

10.0 REFERENCES

U.S. Environmental Protection Agency. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. Office of Research and Development. EPA/600/R-95/136. August 1995

11.0 TEST SUMMARY

Species:	<i>Mytilus</i> spp.
Test Duration:	48 hours
Endpoint:	Normal Alive
Organism Source:	Carlsbad Aquafarms (Carlsbad, CA)
Salinity:	Selection \pm 2‰ recommended
Dissolved Oxygen	>4 mg/L recommended
Test Temperature:	15 \pm 1°C recommended
Dilution water:	1 μ m filtered seawater
Lighting:	16:8 light:dark
Replication:	5 replicates per sample, plus three for water quality
Test Containers:	20 mL glass scintillation vials
Loading:	Approximately 200 embryos per vial in 10 mL
Water Quality:	Dissolved oxygen, pH, salinity, ammonia, and temperature
Reference Toxicant:	Cadmium Chloride (CdCl ₂)

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Acceptability Criteria: Controls: $\geq 70\%$